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β-lactam antibiotic resistance of bacterial isolates from vaginal swab samples of subfertile bitches

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Abstract
Antibiotic resistance is a rising global threat. The inadvertent use of antibiotics resulted in the emergence of bacteria resistant to almost all classes of antibiotics. Objectives of the current study included isolation and identification of aerobic bacteria from the vaginal swabs of subfertile bitches and identification of the resistance exhibited by these isolates against β-lactam class of antibiotics, both by genotypic and phenotypic methods. Antimicrobial susceptibility pattern of the isolates were studied by antibiotic disc diffusion method and detection of antibiotic resistance genes (blaZ, bla_{TEM-1} and bla_{CTX-M}) by polymerase chain reaction (PCR). Bacteria isolated from vagina included *E. coli* (7), *Streptococcus* (5), *Klebsiella* spp. (4), *Staphylococcus* (3), *Enterococcus* (2) *Enterobacter* spp. (1) and *Pasteurella* spp. (1). The isolates manifested high degree of resistance against the seven β-lactam antibiotics employed in the study. The PCR detected the presence of blaZ in five Gram positive isolates and bla_{CTX-M} in five Gram negative isolates. However, bla_{TEM-1} was present in all the Gram negative bacteria isolated. Majority of the bacteria isolated were Gram negative (56.5 per cent). All the bacteria isolated were resistant to atleast two out of the seven β-lactam antibiotics under study. Co-carriage of multiple resistant genes by the same bacteria increased their resistance towards multiple antibiotics. The prevalence of such bacteria in the environment is a great public health concern.

Keywords: Antibiotic resistance, subfertile bitches, aerobic bacteria, antibiogram, blaZ, bla_{TEM-1}, bla_{CTX-M}

1. Introduction
Subfertility in female dogs is a major issue reported by dog breeders lately, especially in breeder owned purebred dogs. In bitches, the condition has been described as the inability to conceive even after single breeding attempt (Bouchard *et al.*, 1991) [6]. Uterine infection is one of the reason implicated for the condition, mainly caused by opportunistic pathogens (Zubair *et al*., 2014) [16]. Several bacterial species present in the normal vaginal flora, at times can act as opportunistic pathogens. Similarity in bacterial species isolated from the vagina of healthy bitches and those with uterine infections (Grundy *et al.*, 2002) [8] ascertained the role of opportunistic pathogens in uterine infections. Inadvertent use of antibiotics, has led to the development of antibiotic resistant superbugs with multidrug resistance property, contributed by various antimicrobial resistant (AMR) genes. β-lactam antibiotics are the most common class of antibiotics used in both animal and human medicine. Hence, the infections caused by AMR bacteria in man and animals, make it difficult for their treatment and cure. The prevalence of such bacteria in companion animals and environment pose a great threat to public health.

2. Materials and Methods
Subfertile bitches (those animals that were not conceived in previous two breeding) in oestrus, presented at the University Veterinary Hospital, Kokkalai, Thrissur, were the study subjects.

2.1 Sample collection and isolation
Aseptically collected vaginal swab samples from twelve subfertile bitches were cultured on 5% cattle blood agar incubated at 37 °C for 24h. MacConkey Agar, Eosin-Methylene Blue Agar and Mannitol Salt Agar were used for the selective isolation of bacteria.

2.2 Identification of bacterial isolates
The isolates obtained were identified based on cultural and morphological characteristics along with biochemical tests, *viz.* catalase, oxidase, indole, methyl red, Voges-Prausker test, citrate, urease, OF test, TSI and sugar fermentation tests (Barrow and Feltham, 1993) [2].

2.3 Antibiotic susceptibility test
Antibiotic sensitivity testing was done by Kirby-Bauer disc diffusion method (Bauer *et al*., 1966) [4] using seven β-lactam antibiotics- amoxicillin, amoxicillin clavulanate (AMC), amoxicillin sulbactam (AMS), ceftriaxone, ceftriaxone sulbactam (CIS), ceftriaxone tazobactam (CIT) and ampicillin (HiMedia).

2.4 DNA extraction
Overnight LB broth culture (at 37 °C) of isolates were used for DNA extraction employing HiPurA Bacterial Genomic DNA Purification Kit, HiMedia, according to manufacturer's instructions.

2.5 PCR detection of β-lactam resistance genes and sequence analysis
The primers and PCR reaction mix are shown in tables 1 and 2. The standardized PCR protocols used for the detection of *blaZ*, bla_{TEM-1} and bla_{CTX-M} are depicted in tables 3, 4 and 5, respectively. PCR master mix was obtained from Takara. The ethidium bromide stained PCR products were electrophoresed in 2 per cent agarose gel by submarine agarose gel electrophoresis in 1X Tris-Borate- EDTA buffer and visualized by gel documentation system (BioRad, USA). One representative amplicon of each gene was sequenced by Sanger's dideoxy method (Eurofins sequencing, Banglore). The obtained sequences were checked by NCBI BLAST analysis.

Table 1: Primers

Gene	Primers	Amplicon size (bp)
*bla*TEM^{-1}	F: 5'-TCGCCGCATACACTATTCTCAGAATGA-3' R: 5'-ACGCTCACCGGCTCCAGATTTAT-3'	445 (Sedighi *et al*., 2017) [12]
bla_{CTX-M}	F: 5'-ATGTGCAGCACCAGTAAAGTGATGGC-3' R: 5'-TGGGTAAAGTAAGTGACCAGAATCAGCGG-3'	593 (Sedighi *et al*., 2017) [12]
blaZ	F: 5' –TACAACTGTAATATCGGAGGG-3' R: 5' CATTACACTCTTGGCGGTTTC-3'	861 (Malik *et al*., 2007) [11]

Table 2: PCR reaction mix

Components	Volume (µL)
Nuclease free water	1.25
Forward primer (10 pM/ µL)	1
Reverse primer (10 pM/ µL)	1
PCR master mix	6.25
Template DNA	3
Total	12.5

Table 3: PCR protocol for *blaZ* gene

Step		Temperature (°C)	Time
Initial denaturation		95	5 min
Denaturation	30 cycles	95	30 sec
Annealing		55	30 sec
Extension		72	1 min
Final extension		72	10 min

Table 4: PCR protocol for bla_{TEM-1} gene

Step		Temperature (°C)	Time
Initial denaturation		95	5 min
Denaturation	30 cycles	95	30 sec
Annealing		63	45 sec
Extension		72	1 min
Final extension		72	10 min

Table 5: PCR protocol for bla_{CTX-M}

Step		Temperature (°C)	Time
Initial denaturation		95	5 min
Denaturation	30 cycles	95	30 sec
Annealing		56.5	45 sec
Extension		72	1 min
Final extension		72	10 min

3. Results and Discussion
3.1 Isolation of bacteria
From twelve vaginal swab samples, 23 bacterial isolates were obtained (Isolates designated as S1a- first isolate of sample no1: S1b- second isolate of sample no. 1 and so on). Isolates identified were *E. coli* (7), *Streptococcus* spp. (5), *Klebsiella* spp. (4), *Staphylococcus* spp. (3), *Enterococcus faecium* (2) *Enterobacter aerogenes* (1) and *Pasteurella multocida* (1) as shown in table 6 (13 Gram negative, 10 Gram positive isolates). Many authors reported similar findings. Bjurstrom (1993) [5] isolated *E. coli*, *Streptococcus*, *Staphylococcus*, *Pasteurella* and *Proteus* from the vagina of bitches with reproductive tract disorders, while Adesokan and Ajala (2011) [1] detected *E. coli*, *Klebsiella*, *Streptococcus pyogenes* and *Staphylococcus aureus* from reproductive tract disorders in bitches. Shambulingappa and Manegar (2010) [13] and Golinska *et al*. (2021) [7] also documented that Gram negative bacteria, especially *E. coli* was the most predominant pathogen in genital tract infections in bitches. Bacteria were isolated either as mixed colonies (58.33%) or as single pure colonies (41.66%).

Table 6: Bacteria isolated from subfertile dogs

Bacterial isolate	No. of isolates	% isolation (n=12)
E. coli	7	58.33
Klebsiella spp.	4	33.33
E. aerogenes	1	8.33
P. multocida	1	8.33
Streptococcus spp.	5	33.33
Staphylococcus spp.	3	25
E. faecium	2	16.67

3.2 Antibiotic sensitivity testing
Antibiotic sensitivity study revealed that all the isolates showed resistance against atleast two of the β-lactam antibiotics under study (Table 7, Fig. 1). Both Gram positive and Gram negative isolates showed highest resistance against amoxicillin and ceftriaxone (95.65 per cent each). Least resistance was shown against ampicillin. Amoxicillin showed highest resistance in isolates obtained from canine pyometra (Unnikrishnan, 2018) [14]. However, Bassessar *et al.* (2013) [3] in a study on antibiogram of bacteria isolated from canine pyometra showed that 55 per cent of isolates were sensitive to amoxicillin and 80 per cent were resistant to ampicillin. The variation in antibiotic susceptibility pattern might be due to development of resistance in the isolates due to indiscriminate use of antibiotics.

Table 7: Anti-microbial resistance of bacterial isolates

Bacterial isolate	No. of isolates	AMX	AMS	AMC	CTR	CIS	CIT	AMP
E. coli	7	7	6	5	7	7	5	3
Klebsiella spp.	4	4	4	3	4	3	4	4
E. aerogenes	1	1	1	1	1	1	1	1
P. multocida	1	0	0	0	1	1	0	0
Streptococcus spp.	5	5	4	5	4	3	3	5
Staphylococcus spp.	3	3	3	3	3	2	2	2
E. faecium	2	2	1	1	2	2	1	1

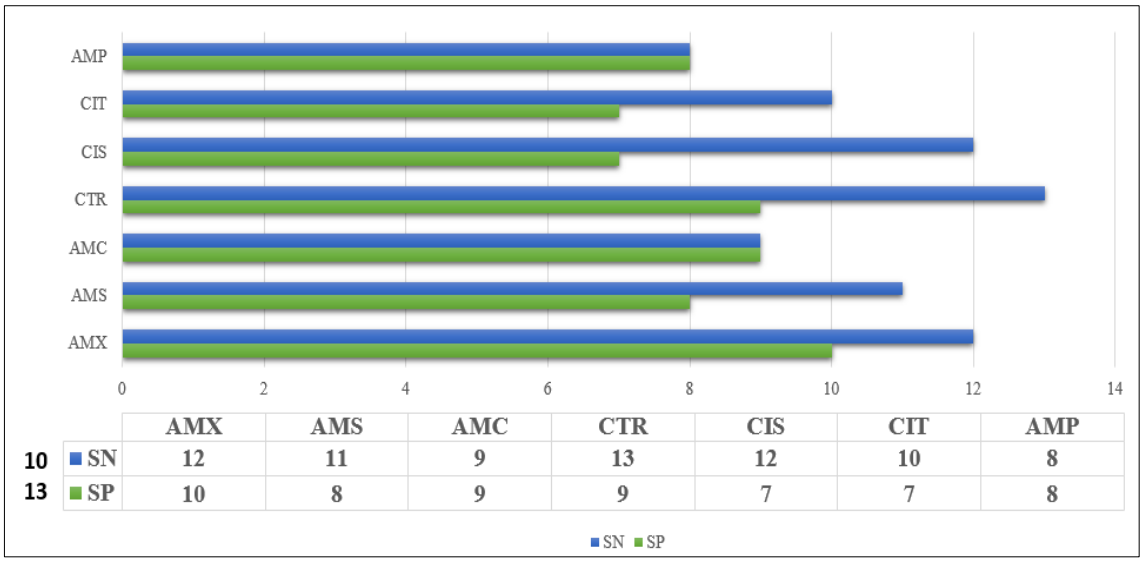

Fig 1: Antibiotic resistance pattern of Gram positive (SP) and Gram negative (SN) isolates

3.3 Molecular detection of antibiotic resistance genes and sequence analysis

PCR results revealed the presence of *bla*$_{TEM-1}$ gene in all 13 and *bla*$_{CTX-M}$ in five of the total 13 Gram negative isolates (Fig. 2 and 4, respectively). *blaZ* was detected only in five of the total 10 Gram positive isolates (Fig. 3). The results are shown in table 8. The results were in accordance with the findings of many studies. Liu *et al.* (2016) [10] and Sedighi *et al.* (2017) [12] observed *bla*$_{TEM-1}$ as the most predominant β-lactam resistance gene in *E. coli* and *Klebsiella pneumoniae*, respectively. Hassan and Abdalhamid (2014) [9] reported that *bla*$_{CTX-M}$ was the most predominant beta-lactamase gene in *Enterobacteriaceae*. *blaZ* gene was the most common β-lactamase gene in canine staphylococci as reported by Malik *et al.* (2007) [11]. Zscheck and Murray (1993) [15] also detected *blaZ* as the major gene involved in the regulation of beta-lactamase production in enterococci and staphylococci.

Sequence analysis of the representative samples confirmed the presence of respective AMR genes.

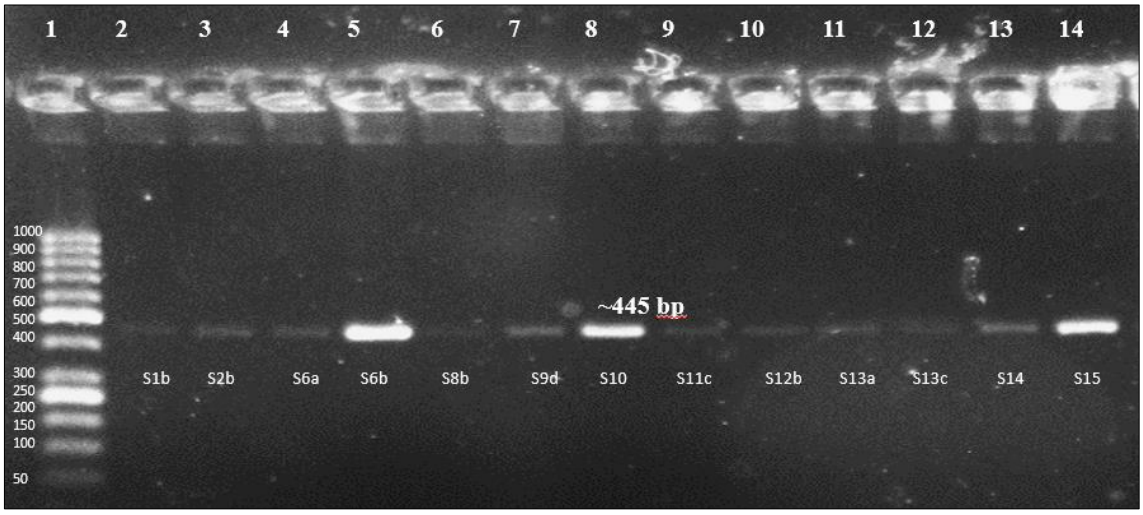

Fig 2: Agarose gel electrophoresis depicting amplicon specific to *bla*$_{TEM-1}$; lane 1- 50bp ladder; lane 2-14- samples

Fig 3: Agarose gel electrophoresis depicting amplicons specific to *blaZ* gene; lane 1- 100bp ladder; lane 2-11- samples

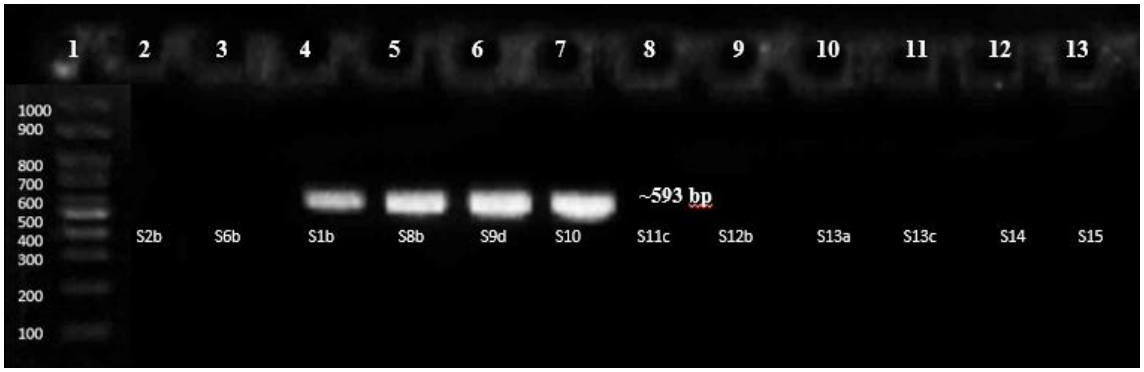

Fig 4: Agarose gel electrophoresis depicting amplicons specific to bla_{CTX-M}; lane 1- 100bp ladder; lane 2-13- samples

Table 8: Prevalence of AMR genes in bacterial isolates

Bacterial isolate	No. of isolates	bla_{TEM-1}	bla_{CTX-M}	*blaZ*
E. coli	7	7	3	-
Klebsiella spp.	4	4	1	-
E. aerogenes	1	1	0	-
P. multocida	1	1	1	-
Streptococcus spp.	5	-	-	3
Staphylococcus spp.	3	-	-	1
E. faecium	2	-	-	1

4. Conclusions

The majority of bacteria isolated from subfertile bitches were Gram negative bacteria, especially *E. coli.* All isolates were highly resistant to β-lactam class of antibiotics. bla_{TEM-1} was the major β-lactam resistant gene in Gram negative bacteria and it was detected in all the isolates. *Escherichia coli* showed the highest prevalence of β-lactam resistant genes than all other Gram negative bacterial isolates, while streptococci showed the highest frequency of *blaZ* gene.

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